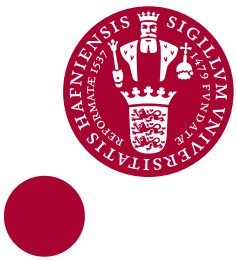

The shady side of leaf development

the role of the REVOLUTA/KANADI1 module in leaf patterning and auxin-mediated growth promotion

Merelo, Paz; Botterweg Paredes, Esther; Heisler, Marcus G.; Wenkel, Stephan

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The shady side of leaf development: the role of the REVOLUTA/KANADI1 module in leaf patterning and auxin-mediated growth promotion

Paz Merelo[1], Esther Botterweg Paredes[2], Marcus G Heisler[1] and Stephan Wenkel[2]

Leaves are present in all land plants and are specialized organs for light harvesting. They arise at the flanks of the shoot apical meristem (SAM), and develop into lamina structures that exhibit adaxial/abaxial (upper/lower side of the leaf) polarity. At the molecular level, an intricate regulatory network determines ad-/abaxial polarity in *Arabidopsis thaliana* leaves, where the Class III Homeodomain Leucine Zipper (HD-ZIPIII) and KANADI (KAN) proteins are key mediators. The HD-ZIPIII REVOLUTA (REV) is expressed in the adaxial domain of lateral organs, whereas KAN1 is involved in abaxial differentiation. The REV/KAN1 module directly and antagonistically regulates the expression of several genes involved in shade-induced growth and auxin biosynthetic enzymes.

Addresses
[1] European Molecular Biology Laboratory (EMBL), 69117 Heidelberg, Germany
[2] Copenhagen Plant Science Centre, Department of Plant and Environmental Sciences, University of Copenhagen, 1871 Frederiksberg C, Denmark

Corresponding authors: Heisler, Marcus G (heisler@embl.de) and Wenkel, Stephan (wenkel@plen.ku.dk)

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Introduction

Leaves are the primary photosynthetic organs of vascular plants, enabling them to convert CO_2 into sugars for energy storage. The shape of leaves is usually flattened, presumably reflecting a common selective pressure for maximizing light capture while minimizing weight. However, leaves are not only flat but also consist of distinct layers of cell types that form their upper (adaxial) and lower (abaxial) tissues with the boundary between these tissues located in the middle. While at maturity these tissues are specialized for photosynthesis and gas exchange, respectively, during leaf development these cell types also play a critical role in establishing the final flat shape of the leaf. In Arabidopsis, when genes that promote adaxial or abaxial tissue identity are ectopically expressed or reduced in function, the resulting disruptions to tissue identity correlate with dramatic changes in leaf morphology. For instance, ectopic expression of the Class III family of HD-ZIP transcription factors REVOLUTA, PHABULOSA and PHAVOLUTA results in organs consisting of predominantly adaxial cell types and the resulting leaves are radially symmetric (centric) rather than flattened [1,2]. Similarly, leaf primordia that are abaxialized due to ectopic expression of the *KANADI* (*KAN*) genes also develop in a centric manner [2,3,5]. These observations demonstrate both adaxial and abaxial tissues need to be present for leaves to develop a lamina shape. Consistent with this, genes involved in maintaining lamina growth and integrity, such as the *WUSCHEL RELATED HOMEOBOX* (*WOX*) genes, are expressed at the ad-/abaxial boundary [6].

Apart from shaping their leaves appropriately, plants also maximize their light capture by modifying leaf position and overall stature to avoid shading by neighbouring plants, which is collectively called the shade avoidance response. As it turns out, many of the same genes involved in shaping leaves in seed plants are also involved in mediating shade avoidance responses [7,8•,9,10•]. However, this finding is perhaps not so surprising given that both processes involve the regulation of biosynthesis, transport and signalling of auxin. In this review, we will detail recent findings on similarities between the core regulatory networks underlying both leaf ad-/abaxial polarity and shade response, and highlight recent data relating these networks to auxin.

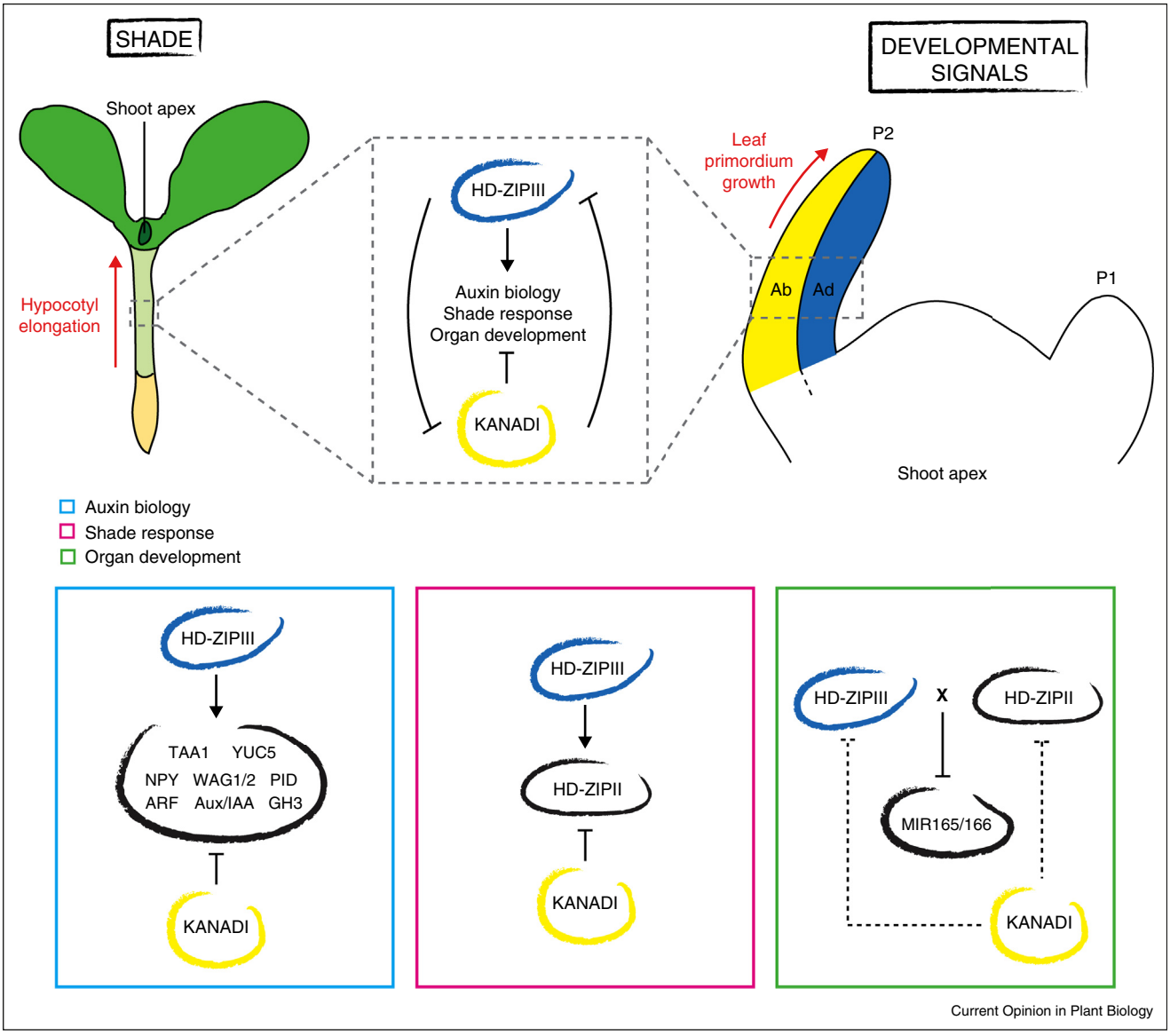
The HD-ZIPIII/KAN module regulates adaxial/abaxial cell identity and shade-induced growth

Leaves initiate as primordia on the flanks of the shoot apical meristem. The leaf primordium is composed of adaxial and abaxial tissues that give rise to future upper and lower tissues of the leaf. Here, primordium tissue situated closer to the shoot apical meristem (SAM) is referred to as the adaxial domain while primordium tissue on the opposite side, facing away from the SAM is called the abaxial domain. Members of the *HD-ZIPIIIs* are expressed in

the adaxial domain where they act as master regulators of adaxial cell identity [1,2]. In the abaxial domain, members of the KAN family of transcription factors literally mirror *HD-ZIPIII* expression and act as regulators of abaxial cell identity [2,3]. Besides their complementary patterns of expression, the study of loss- and gain-of-function mutations in *HD-ZIPIII* and *KAN* genes has revealed an antagonistic relationship between them [2,11,12].

REV and KAN1 directly regulate a number of genes or genetic pathways in an opposite manner [7,13••,14•], suggesting that the REV/KAN1 antagonism relies partly on the opposite regulation of common biochemical pathways (Figure 1). In part this also reflects the finding that REV acts mainly as a transcriptional activator while KAN1 tends to act as a transcriptional repressor [7,8•,13••,14•]. In addition to genes involved in organ development and adaxial/abaxial patterning, several joint target genes encode regulators of shade response such as HD-ZIPII transcription factors as well as TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1) and YUCCA5 (YUC5), which are involved in

Figure 1



The HD-ZIPIII/KAN module regulates shade avoidance and leaf development. Top row: HD-ZIPIII and KAN proteins display polar expression patterns in the vasculature of the hypocotyl and in the leaf primordium. HD-ZIPIIIs (REV) and KANs (KAN1) oppositely regulate genes involved in auxin biology, shade avoidance response and organ development, which may drive both hypocotyl elongation and leaf growth. P1 is the youngest leaf primordium and P2 is the next oldest leaf primordium. Bottom row: Genetic interactions between REV, KAN1 and genes involved in auxin biosynthesis, transport and signalling (blue box), shade-avoidance (magenta box) and organ development (green box).

the production of auxin [15,16]. This implies a connection of the regulatory network involved in leaf patterning and the network operating in the shade response pathway (Figure 1).

Regulation of auxin biology

Both the synthesis and transport of auxin influence plant organ polarity [11,17]. Auxin is in part produced in a two-step process: TAA alliinase enzymes [18,19] convert the amino acid tryptophan to IPA (indole-3-pyruvate) from which YUCCA-type (YUC) flavin monoxygenase enzymes produce IAA (indole-3-acetic acid), the most abundant auxin in plants [20]. Both REV and KAN1 regulate *TAA1* and YUC5, which constitute the linear tryptophan-dependent auxin production pathway [7,8•] and in agreement with previous findings, ectopic expression of REV causes up-regulation of *TAA1* and *YUC5* and an increase in auxin production whereas ectopic expression of KAN1 represses both *TAA1* and *YUC5*, which results in low auxin levels. Both higher order *taa/tar* mutant plants [18] as well as higher order *yuc* mutants display defects in leaf blade expansion and the latter have also been associated with leaf margin development and blade outgrowth [21]. Besides the regulation of auxin biosynthesis, the REV/KAN1 module seems to impinge on a number of genes encoding auxin signalling components [13••,14•], including several *AUXIN RESPONSE FACTOR* (*ARF*), *SMALL AUXIN UP-REGULATED* (*SAUR*), *GH3* and *Aux/IAA* genes, and auxin transport components such as *PIN-FORMED* (*PIN*), *PINOID* (*PID*), *PID* homolog (*WAG1/2*) and *NAKED PINS IN YUC MUTANTS* (*NPY*) genes [22,23,24,25,26]. While not all these genes are common targets, REV and KAN1 often regulate different genes within a common pathway. For instance, although only KAN1 has been shown to regulate *PIN1* and *PID*, both transcription factors regulate members of the WAG and NPY gene families. Thus, auxin transport is a common target. Overall these findings suggest that the REV/KAN1 module affects not only the production of auxin but also its transport and downstream signalling (Figure 1). However, of these genes, so far only *ARF3* and *TAA1* have been shown to be differentially expressed along the adaxial-abaxial axis during leaf development [7,27,28]. In fact, it is not clear at this point how the opposite regulation of auxin by REV and KAN1 relates to organ initiation since low levels of auxin are required in adaxial tissues to promote adaxial cell fate [29].

To avoid growing in the canopy of other vegetation, plants can sense the red/far-red ratio with their phytochrome systems and induce elongation growth. A high red/far-red (R:FR) ratio, is e.g. found in an open field environment. Here, elongation growth is suppressed through the direct binding and inhibition of PHYTOCHROME INTERACTING FACTOR (PIF) transcriptional regulators by active phytochrome B (PHYB). This contrasts shade conditions, where the R:FR ratio is low due to far-red reflection from neighbouring vegetation. In this situation, PHYB is in its inactive state and unleashes the PIF factors to promote elongation growth [30]. The shade-induced wave of transcription is followed by a boost in auxin biosynthesis [31] that is required for elongation growth. Plants carrying either loss-of-function mutations in the *TAA1* gene or higher order *yucca* mutants (*yuc1 yuc4* and *yuc1 yuc2 yuc6*) remain short in shade conditions, do not produce high levels of auxin and are thus more shade insensitive [19,20]. The production of auxin initiates following the breakdown of cytokinine in young leaf primordia, which prevents the continued growth of developing leaves. It is assumed, that this inhibition of growth releases resources that can be redirected to the growing hypocotyl [32].

The analysis of *hd-zipIII* and *kanadi* loss- and gain-of-function mutants has revealed the involvement of the HD-ZIPIII/KAN module in shade-induced growth promotion [7,33]. Loss of *HD-ZIPIII* function (as in *rev* mutants or in plants with ectopic *MIR165a* expression) results in reduced elongation growth in response to shade while REV gain-of-function (*rev10D*) mutants have slightly elongated hypocotyls under non-shade conditions. Ectopic expression of KAN1, causes a complete suppression of shade-induced growth and *kan1 kan2* double mutants have, like *rev10D*, elongated hypocotyls in white light conditions [8•]. However, in comparison to *rev10D*, which shows normal hypocotyl elongation in shade, *kan1 kan2* double mutants show a significantly reduced response [8•]. These findings support a model that relies on the opposing activities of HD-ZIPIIIs and KANs for allowing elongation growth in response to shade (Figure 1).

Within the hypocotyl, both REV and TAA1 are expressed in the inner cylinder of the vascular system [7,28]. The PIN3 auxin efflux carrier, which is absolutely required for a full shade avoidance response, is expressed in the endodermis and the epidermis, and changes its localization pattern in response to shading from basal to a more lateral position [34]. It is currently unclear how auxin production, transport and signalling are regulated across the different hypocotyl cell types but a recent study suggests an important role for the epidermis in directing hypocotyl growth [35•].

Regulation of HD-ZIPIIs

The analysis of direct downstream target genes of REV identified several genes encoding HD-ZIPII transcription factors that are directly activated by REV [7]. These *HD-ZIPII* genes including HOMEOBOX ARABIDOPSIS THALIANA 2 (*HAT2*), *HAT3*, ARABIDOPSIS THALIANA HOMEOBOX 2 (*ATHB2*) and *ATHB4*, are all known to promote growth in response to shade and are part of the first wave of transcriptional up-regulation [36,37,38]. In the absence of these transcription factors,

e.g. in the *hat3 athb4* double mutant, hypocotyl elongation is impaired [39]. Moreover, higher order *hd-zipII* mutants, such as *hat3 athb4* and *hat3 athb2 athb4* exhibit additional embryo and leaf polarity defects strongly resembling higher order *hd-zipIII* mutants [9,10•]. Indeed, the *cis*-element recognized by HD-ZIPII and HD-ZIPIII transcription factors share the same core sequence [AAT(G/C)ATT] [40] suggesting that HD-ZIPII/IIIs may redundantly regulate the same targets. However, in contrast to the HD-ZIPIIIs, all the HD-ZIPII proteins contain an Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) domain, which suggests they interact with TOPLESS/TOPLESS-LIKE co-repressor proteins [41,42] and to likely act as transcriptional repressors [10•,38,43]. This further implies that HD-ZIPIIs may function either as repressors of genes activated by REV, or as repressors of factors that restrict HD-ZIPIIIs [44•]. In either case, HD-ZIPIIIs would be affected, resulting in higher HD-ZIPIII activity in situations of high HD-ZIPII expression or in reduced HD-ZIPIII activity as seen in *hat3 athb4* and *hat3 athb2 athb4* mutant plants [9]. Interestingly, in a recent study, it was revealed that the HD-ZIPIII transcription factor REV, whose expression is restricted to the adaxial side of the leaf by the activity of the microRNAs miR165/166, physically interact with their targets the HD-ZIPII proteins HAT3 and ATHB4 to directly repress *MIR165/166* expression in the adaxial domain [45••]. In particular, such direct repression is established via a previously characterized *cis*-element located close to the *MIR165/166* genes [45••,46]. These results unveil at least one of the molecular functions of both HD-ZIPII and HD-ZIPIII proteins in the establishment of leaf polarity and suggest the possibility that they may also act together to similarly regulate shade response-related genes, which would be an interesting challenge to address in the future.

Besides the HD-ZIPII/HD-ZIPIII interaction, the HD-ZIPII members *HAT1* and *HAT2*, which are involved in shade-avoidance response [7,39], have been shown to be down-regulated by ectopic KAN1 under shade conditions [7,8•,13••]. These findings also support the shared REV/KAN1 common targets hypothesis and connect the developmental and shade-avoidance regulatory networks (Figure 1).

Feedback regulation between shade perception and leaf development

Shade has a profound impact on plant development, especially for the leaves. When grown in shade, Arabidopsis leaves show characteristic features such as elongated petioles and a smaller and thinner leaf blade. The molecular processes underlying these changes are however not well understood. Nevertheless, different photoreceptor mutants exhibit characteristic leaf growth defects suggesting that light and the downstream signalling cascade play an additional role in leaf patterning.

Phytochromes and phototropins are photoreceptors that capture the red/far-red, and blue spectrum of light, respectively. Members of both the phytochrome and phototropin family have been shown to antagonistically control leaf curling. Plants carrying loss-of-function mutations in both *PHOTOTROPIN 1* (*PHOT1*) and *PHOT2* display strongly downward curled leaves that resemble *hd-zipIII* loss-of-function mutants [47••]. The addition of a further mutation in the *PHYB* photoreceptor rescues the downward curling phenotype and produces a flat lamina [47••]. These findings support a role for red/far-red light in patterning Arabidopsis leaves.

How and at which developmental stage shade affects leaf development is still largely unknown but studies on leaf development in tomato have provided some first insights. In response to shading, the area of tomato leaves exhibits great plasticity, being shade-responsive also late in development [48••]. However other features such as stomatal index, which is the ratio of the number of stomata per epidermal pavement cells, can only be affected in the early stages of leaf development. In response to continuous shade treatments, an increase in both the size of the SAM and incipient leaf primordia was observed. Further gene expression profiling studies revealed profound changes in the expression of *KNOTTED1*-like homeobox (KNOX) and KNOX-related genes in young leaf primordia that impinge on known patterning pathways [48••].

To summarize, there is increasing evidence that shade growth and leaf patterning share common regulatory modules in which auxin seems to play a decisive role. However, a future challenge will be to dissect these regulatory connections at cellular resolution and further test them functionally.

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47. •• Kozuka T, Suetsugu N, Wada M, Nagatani A: **Antagonistic regulation of leaf flattening by phytochrome B and phototropin in Arabidopsis thaliana**. *Plant Cell Physiol* 2013, **54**:69-79.
This study shows that leaf flatness is controlled by photoreceptors. In comparison to wild type plants, phyB mutants have flatter leaves while overexpression of PHYB causes severe downward curling. Also plants deficient in the two blue light photoreceptors PHOT1 and PHOT2 have downward-curled leaves and this phenotype is rescued in a phyB mutant background. Thus PHYB and PHOT1/2 control leaf flatness in an antagonistic manner.

48. •• Chitwood DH, Kumar R, Ranjan A, Pelletier JM, Townsley BT, Ichihashi Y, Martinez CC, Zumstein K, Harada JJ, Maloof JN *et al.*: **Light-induced indeterminacy alters shade-avoiding tomato leaf morphology**. *Plant Physiol* 2015, **169**:2030-2047.
This study provides first insights about how and at what developmental stage shade affect leaf development in tomato. In leaf primordia, shade avoidance is mediated through the expression of KNOTTED1-LIKE HOMEOBOX and other indeterminacy genes, affecting known developmental pathways involved in patterning leaf shape.